DNA Polymerase β Inhibitors from *Baeckea gunniana*

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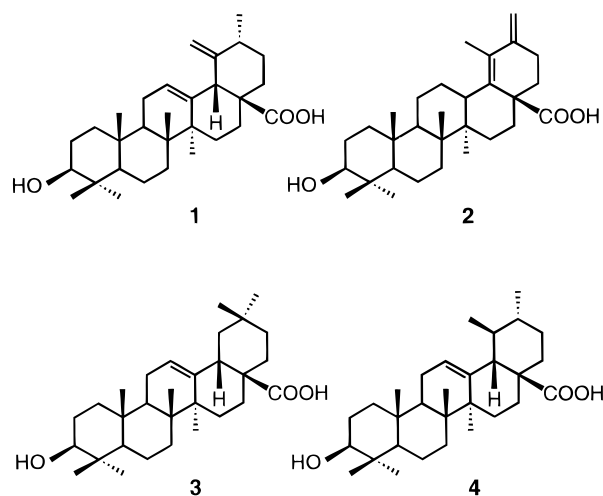
Crude plant extracts were surveyed for their ability to inhibit DNA polymerase β. A methyl ethyl ketone extract prepared from *Baeckea gunniana* was identified as a potent inhibitor of the enzyme. Bioassay-guided fractionation of the extract, using an assay to monitor the inhibitory potential of individual fractions toward DNA polymerase β, led to the isolation of four active ursane and oleanane triterpenoids (**1**–**4**). Inhibitory principle **1** is a new natural product, and **2** is a novel compound. Their structures were established as 3β-hydroxyurs-12,19(29)-dien-28-oic acid (**1**) and 3β-hydroxyurs-18,20(30)-dien-28-oic acid (**2**) by spectroscopic analysis and by comparison with the data for the structurally related compound ursolic acid (**4**). Also isolated as a DNA polymerase β inhibitor was oleanolic acid (**3**). Compounds **1**–**4** had IC_{50} values of 5.3–8.5 μM as inhibitors of polymerase β in the presence of bovine serum albumin (BSA) and 2.5–4.8 μM in the absence of BSA.

DNA polymerase β, a 39 kDa gap-filling enzyme involved in base excision repair,[1–3] is responsible for repairing damaged DNA after exposure to such chemotherapeutic agents as monofunctional DNA alkylation agents,[2] cisplatin,[3] bleomycin,[4,5] and neocarzinostatin.[5] Due to its central role in DNA repair, DNA polymerase β is a potential target for adjuvant antitumor therapy; selective inhibition of this enzyme by otherwise noncytotoxic agents could possibly potentiate chemotherapeutic treatment by DNA-damaging agents, thus improving the efficacy of anticancer drugs and permitting lower doses to be administered. Indeed, our recent study using isolated DNA polymerase β inhibitors indicated that inhibition of DNA polymerase β in cultured cells resulted in potentiation of the cytotoxity of bleomycin and cisplatin.[6] Naturally occurring DNA polymerase β inhibitors characterized to date have included bis-5-alkylresorcinols,[7] a diterpenoid-substituted methylhydroquinone,[8] lanostane-type triterpenoids,[9,10] and flavonoids,[11] as well as fatty acids[12] and their derivatives.[6,13]

In our continuing survey of crude plant extracts to identify DNA polymerase β inhibitors, we found that a methyl ethyl ketone extract prepared from *Baeckea gunniana* Schau. ex Walp. (Myrtaceae) exhibited potent inhibition of DNA polymerase β (78% inhibition at 100 μg/mL; 69% inhibition at 50 μg/mL). Accordingly, the crude extract was subjected to fractionation, using an assay to monitor DNA polymerase β inhibition, to permit isolation and characterization of the principle(s) responsible for inhibition of the enzyme. The bioassay-guided fractionation of the crude extract led to the isolation of four DNA polymerase β inhibitory pentacyclic triterpenoids (**1**–**4**). Inhibitory principle **1** is a new natural product, and **2** is a novel compound. Reported herein is the isolation of inhibitors **1**–**4** through bioassay-guided fractionation and the determination of their structures as well as their potencies as DNA polymerase β inhibitors.

Results and Discussion

The twigs and leaves of *B. gunniana* were soaked successively with hexanes, methyl ethyl ketone, methanol, and water. The methyl ethyl ketone extract was found to inhibit DNA polymerase β (Table 1 in the Supporting Information) and was fractionated initially on a polyamide 6S column, which was washed successively with H_2O, 1:1 $MeOH-H_2O$, 4:1 $MeOH-CH_2Cl_2$, 1:1 $MeOH-CH_2Cl_2$, and 9:1 $MeOH-NH_4OH$. The final eluate was strongly inhibitory to enzyme activity, presumably because this fraction contained polyphenols, which tend to bind DNA strongly. The 4:1 $MeOH-CH_2Cl_2$ fraction had significant DNA polymerase β inhibitory activity (84% inhibition at 100 μg/mL; 70% inhibition at 50 μg/mL) and was applied to a Sephadex LH-20 column for further fractionation employing a normal-phase elution scheme. The 1:1 $CH_2Cl_2-Me_2CO$ fraction from the Sephadex LH-20 column, which showed the strongest inhibition, was fractionated further using a C_8 reversed-phase open column. Two fractions (13:7, and 18:2 $MeOH-H_2O$) from the C_8 open column had the greatest inhibitory activity. These two fractions were combined and applied to a C_{18} reversed-phase HPLC column for further fractionation. The three active fractions from the HPLC column afforded inhibitory principles **1**–**4** after further purification by C_{18} reversed-phase HPLC.



Compounds **3** and **4** were identified as oleanolic acid and ursolic acid, respectively, by direct comparison (^1H, ^{13}C NMR[14] and $[\alpha]_D$ data[15]) with authentic samples. Compounds **1** and **2** were obtained as colorless powders. The molecular formula ($C_{30}H_{46}O_3$) for **1** was determined based

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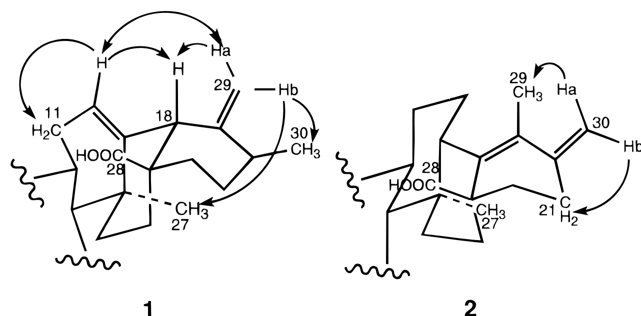

Figure 1. NOE interactions of compounds **1** and **2**.

on the $[M + Li]^+$ ion observed at *m/z* 461.3607 ($C_{30}H_{46}O_3$-Li) in the HRFABMS; this indicated the presence of two fewer hydrogen atoms in **1** than in **4.** The ^{1}H NMR spectrum of **1** was very similar to that of **4**, except for the absence of a doublet methyl signal at δ 0.82 (3 H, d, $J =$ 6.5 Hz) and the presence of two additional terminal olefinic proton signals at δ 4.47 and 4.52 (each br s). The carbon signals at δ 104.3 and 152.5 in the ^{13}C NMR spectrum of **1** (Table 1) also supported the presence of a vinylic double bond in the structure. Based on the analysis of the ^{1}H and ^{13}C NMR data, there could be two possible assignments for the position of the vinylic group, either C-20(30) or C-19-(29). Although the proton peaks at ca. δ 2.10 were heavily overlapped, the signal due to H-18 at δ 2.14 could be distinguished as a singlet, suggesting that **1** has a C-19-(29)-ene structure. This was confirmed through NOE difference measurements involving certain resonances.[16] Irradiation at δ 5.13 (H-12) resulted in NOEs at δ 2.14 (s, H-18) and 4.52 (br s, Ha-29); irradiation at δ 4.52 gave NOEs at δ 2.14 (s, H-18) and 5.13 (t, H-12), and irradiation at δ 4.47 (Hb-29) afforded NOEs at δ 0.84 (CH_3-30) and 0.99 (CH_3-27) (Figure 1). The NOE interaction between the vinylic proton and CH_3-27 also confirmed the β structure configuration of H-18; that is the D/E ring junction is cis.[16] Accordingly, the structure of **1** was established as 3 β-hydroxyurs-12,19(29)-dien-28-oic acid. Although compound **1** has been reported previously as a synthetic product,[17] the present finding constitutes its first isolation from a natural source.

Compound **2** had the same molecular formula ($C_{30}H_{46}O_3$) as **1** as judged by the HRFABMS. The ^{1}H NMR spectrum of **2** was quite similar to that of **1** and **4**, except for the absence of the olefinic proton signal corresponding to H-12 (δ 5.13), the absence of the doublet methyl signal corresponding to CH_3-29 in **4**, and the presence of an additional singlet methyl group at δ 1.48 (s), presumably adjacent to a double bond. The ^{13}C NMR spectrum of **2** (Table 1) showed four olefinic carbons at δ 151.7, 151.6, 148.1, and 109.6, indicating that there was a conjugated, tetrasubstituted double bond in **2** in addition to the vinylic group. Combined analysis of the ^{1}H and ^{13}C NMR data indicated the only possible position for the two conjugated double-bond groups as a C-18,20(30)-diene in **2**. The NOE difference experiments as shown in Figure 1 also confirmed the assignment. Accordingly, the structure of **2** was established as 3β-hydroxyurs-18,20(30)-dien-28-oic acid.

Compounds **1**−**4** exhibited strong inhibitory activity toward rat DNA polymerase β, with IC_{50} values of 5.3, 5.6, 7.5, and 8.5 μM, respectively, in the presence of bovine serum albumin (BSA). The corresponding values were 3.2, 2.5, 3.7, and 4.8 μM in the absence of BSA (Table 2). Their inhibitory activitites toward DNA polymerase β were thus not greatly affected by the presence of serum albumin, a basic protein known to bind many lipophilic and acidic

Table 1. ^{13}C NMR Data for Compounds **1**, **2**, and **4** ($CDCl_3$ + CD_3OD, 75 MHz)

carbon	1	2	4
1	38.9	38.8	38.9
2	27.4	27.8	27.5
3	78.9	79.1	78.6
4	38.2	38.3	38.4
5	54.8	56.2	55.2
6	17.8	16.9	18.1
7	32.5	34.3	33.2
8	38.2	40.6	39.1
9	47.0	47.0	47.2
10	36.8	37.1	37.0
11	15.6	15.7	16.1
12	125.4	32.3	125.3
13	137.3	50.6	138.2
14	41.6	42.3	41.9
15	29.2	30.5	28.5
16	23.7	25.4	24.4
17	47.5	48.5	48.2
18	54.1	151.6[a]	52.8
19	152.5	148.1	39.2
20	38.4	151.7[a]	38.8
21	31.1	30.5	30.6
22	36.4	37.1	36.8
23	27.4	27.8	27.9
24	14.8	14.5	15.3
25	15.0	15.1	15.8
26	17.8	19.1	17.8
27	22.9	25.4	23.4
28	179.2	178.5	179.3
29	104.3	21.0	23.8
30	22.8	109.6	21.9

[a] Assignments may be reversed.

Table 2. DNA Polymerase β Inhibitory Activity for Pentacyclic Triterpenoids from *Baeckea gunniana*

	IC_{50} (μM)	
compound	in the presence of BSA[a]	in the absence of BSA[a]
1	5.3	3.2
2	5.6	2.5
3	7.5	3.7
4	8.5	4.8

[a] Bovine serum albumin.

species;[18] this is consistent with the possibility that these inhibitors may be of utility in vivo. Inhibitors **1** and **2**, which both contain an exocyclic double bond on their E rings, displayed slightly enhanced inhibitory potential. Although some ursane and oleanane triterpenoids have been reported to exhibit cytotoxic activity against KB cells[19] or P-388 lymphocytic leukemia cells,[20] these are the first examples of compounds in the ursolic and oleanolic acid series that potently inhibit DNA polymerase β.

Experimental Section

General Experimental Procedures. Polyamide 6S (a product of Riedel-de Haen, Germany) was purchased from Crescent Chemical Co. Sephadex LH-20 (Pharmacia; 40 μm) was obtained from Sigma Chemicals. Silica reversed-phase C_8 resin (32−60 μm) was obtained from ICN Pharmaceuticals. The Kromasil reversed-phase C_{18} HPLC column (250 × 10 mm, 5 μm) for HPLC was from Higgins Analytical, Inc. Optical rotations were measured on a Perkin−Elmer 243B polarimeter. ^{1}H and ^{13}C NMR spectra were obtained on a General Electric GN-300 or QE-300 NMR spectrometer. HRFABMS were recorded on a VG ZAB−SE mass spectrometer. Calf thymus DNA and unlabeled dNTPs were purchased from Sigma Chemicals; [^{3}H]dTTP was purchased from ICN Pharmaceuticals. DEAE−cellulose paper (DE-81) was from Whatman.

Plant Material. Twigs and leaves of *Baeckea gunniana* were collected in Tasmania in January 1973. A voucher specimen (IJE-3131) is stored at the U.S. National Aboretum, Herbarium, Washington, DC.

Extraction and Isolation. Twigs and leaves of *B. gunniana* were soaked successively with hexanes, methyl ethyl ketone, MeOH, and H_2O. The methyl ethyl ketone extract exhibited inhibitory activity toward DNA polymerase β (78% inhibition at 100 μg/mL; 69% inhibition at 50 μg/mL). The crude extract retained significant inhibitory activity after passage through a polyamide 6S column to remove polyphenols. Therefore, this crude extract was chosen for bioassay-guided fractionation. A total of 857 mg of methyl ethyl ketone crude extract was used for the bioassay-guided fractionation; a typical set of experiments is described below. The crude extract (286 mg) was fractionated initially on a (10-g) polyamide 6S column, which was washed successively with 150-mL portions of H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and 9:1 MeOH−NH_4OH. The 4:1 MeOH−CH_2Cl_2 fraction (143 mg) strongly inhibited DNA polymerase β (84% inhibition at 100 μg/mL) and was fractionated further on a (15-g) Sephadex LH-20 column that was eluted successively with 250-mL portions of hexane, 1:1 hexane−CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2−Me_2CO, Me_2CO, and MeOH. The 1:1 CH_2Cl_2−Me_2CO fraction (57 mg), which showed the strongest inhibitory activity (78% inhibition at 50 μg/mL), was applied to a C_8 reversed-phase open column for further fractionation, using 11:9, 13:7, 15:5, 17:3, 18:2, 19:1, and 20:0 MeOH−H_2O as eluents. The 17:3 and 18:2 MeOH−H_2O fractions displayed the strongest inhibition of DNA polymerase β and were combined. The combined fraction (21 mg) was then applied to a C_{18} reversed-phase HPLC column (250 × 10 mm, 5 μm) and washed with a linear gradient of 4:1→19:1 CH_3CN−H_2O over a period of 50 min at a flow rate of 2.5 mL/min (monitoring at 220 nm). Three strongly active fractions were obtained from the C_{18} HPLC column. Purification of these three fractions employing the same C_{18} reversed-phase HPLC column and eluting with 90% CH_3CN in H_2O at a flow rate of 2.0 mL/min (monitoring at 220 nm) afforded purified active compounds **1** (1.1 mg), **2** (0.6 mg), **3** (1.0 mg), and **4** (7 mg).

Compound 1: colorless powder $[\alpha]^{22}_D$ + 48° (*c* 0.2, MeOH); (partial) ^{1}H NMR ($CDCl_3$ + 5% CD_3OD, 300 MHz) δ 0.59 (3H, s), 0.62 (3H, s), 0.71 (3H, s), 0.80 (3H, s), 0.84 (3H, d, J = 4.8 Hz, CH_3-20), 0.99 (3H, s), 2.14 (1H, s, H-18), 3.02 (1H, t, J = 9.6 Hz, H-3), 4.47 (1H, br s, Hb-29), 4.52 (1H, br s, Ha-29), 5.13 (1H, t, J = 3.5 Hz, H-12); ^{13}C NMR, see Table 1; HRFABMS *m/z* 461.3607 [M + Li]$^+$ (calcd for $C_{30}H_{46}O_3Li$, 461.3607).

Compound 2: colorless powder; $[\alpha]^{22}_D$ + 32° (*c* 0.15, MeOH); (partial) ^{1}H NMR ($CDCl_3$ + 5% CD_3OD, 300 MHz) δ 0.52 (3H, s), 0.61 (3H, s), 0.76 (6H, s), 0.78 (3H, s), 1.48 (3H, s, CH_3-29), 2.94 (1H, t, J = 9.2 Hz, H-3), 4.57(1H, br s, Ha-30), 4.71 (1H, br s, Hb-30); ^{13}C NMR, see Table 1; HRFABMS *m/z,* 477.3324 [M + Na]$^+$ (calcd for $C_{30}H_{46}O_3Na$, 477.3345).

DNA Polymerase β Inhibition Assay. After dissolving the crude extract samples or fractions in 1:1 DMSO−MeOH, 6 μL of the sample and 4 μL of rat DNA polymerase β preparation[21] (6.9 units, 48 000 units/mg) were added to 50 μL of 62.5 mM 2-amino-2-methyl-1,3-propanediol buffer, pH 8.6, containing 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL BSA, 6.25 μM dNTPs, 0.04 Ci/mmol [^{3}H]dTTP, and 0.25 mg/mL activated calf thymus DNA. After incubation at 37 °C for 1 h, the radioactive DNA product was collected on DEAE−cellulose filters and dried. The radioactive filters were washed successively with 0.4 M K_2HPO_4, pH 9.4, and 95% EtOH and then used for determination of radioactivity.

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Supporting Information Available: Table 1: Bioassay data for intermediate fractions and purified polymerase β inhibitors. Proton NMR spectra for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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